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Expression of cyclin E in colorectal adenomas and adenocarcinomas: correlation with expression of Ki-67 antigen and p53 protein

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Abstract The expression of cyclin E in human colorectal adenomas and adenocarcinomas was examined immunohistochemically to elucidate the role of cyclin E in the colorectal carcinogenesis. The expression of cyclin E was detected in 25% (91/358) of the adenomas and 56% (149/267) of the adenocarcinomas. The incidence of strongly positive cases was significantly higher in the adenocarcinomas (20%) than in the adenomas (5%) ($P<0.01$). Among adenomas, a significant correlation was noticed between the expression of cyclin E and the grade of atypia. The incidence of cyclin E expression was significantly higher in the adenocarcinomas without an adenoma component (62%; 104/169) than in those with this component (46%; 45/98) ($P<0.05$). Furthermore, the incidence of the cyclin E expression was higher in stages 1 and 2 carcinoma than in stage 0 and stages 3 and 4 carcinoma. The expression of cyclin E was the most prominent in tumors invading the submucosa and muscularis propria. The expression of cyclin E was significantly correlated with the proliferative activity of the tumor cells measured by Ki-67 antigen expression ($P<0.01$). It was also correlated with the expression of p53 protein in the tumor cells ($P<0.01$). Overexpression of cyclin E and subsequent deregulation of cell cycle may contribute to the development and early progression of the colorectal carcinomas.

Key words Cyclin E · Colorectal adenoma · Colorectal carcinoma · Immunohistochemistry · Ki-67 · p53

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Introduction

Progression of cell cycle is mediated by multiple cyclins and cyclin-dependent kinases (CDKs) [9, 22]. In mammalian cells, key regulators of G1 progression include cyclin E, which combines later in G1 with CDK2, and cyclin D (D1, D2 and D3), which assemble with either CDK4 and CDK6 [9, 22]. Recent studies have identified additional regulators for the CDKs, such as $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{MTS1}$ and $p15^{MTS2}$, which bind to the cyclin–CDK complex and inhibit its kinase activity [5–7, 12, 18, 19, 21, 23]. Therefore, alterations in cell cycle regulators and subsequent deregulation of the G1/S transition may cause uncontrolled cell cycle progression and may implicate in the development and progression of cancer.

Increased expression of various cyclins has been noted in human cancers [9]. In particular, the connection between cyclin D1 and cancers has been studied in some detail [9]. Cyclin D1 gene is amplified in carcinomas of the head and neck, esophagus and breast [3, 11, 20, 29], and in these cancers, gene amplification and overexpression of cyclin D1 correlate well with the malignant behavior of cancer and the prognosis [29]. However, cyclin E is the most prominent of the G1 cyclins and is crucial in the initiation of DNA replication [8]. Overexpression of cyclin E has been observed in various human carcinoma cell lines and tumor tissues including breast cancer, in some cases as a result of cyclin E gene amplification [3, 13, 14, 17]. Altered expression of cyclin E becomes more severe with tumor stage and grade and is consistent with proliferative activity [4, 14]. We have recently found that 10–15% of gastric and colorectal carcinomas show gene amplification of cyclin E [1, 15], and carcinomas with cyclin E gene amplification reveal overexpression of cyclin E. In gastric carcinomas, lymph node metastasis is correlated with cyclin E gene amplification [1]. However, no study has been demonstrated the expression of cyclin E in adenomas and adenocarcinomas of the colorectum.

In the present study, we examined the expression of cyclin E immunohistochemically in 358 adenomas and 267 adenocarcinomas of the colorectum. We also ana-

lyzed the correlation of cyclin E expression with clinicopathological findings and with the expression of Ki-67 and p53 to elucidate the role of cyclin E in the progression of colorectal carcinomas.

Materials and methods

A total of 267 adenocarcinomas and 358 adenomas of the colorectum were studied. They were obtained by surgery, endoscopic removal and biopsy. They were fixed in 10% buffered formalin and embedded in paraffin. One or two representative slides, including both central and peripheral area in advanced cases, were analyzed from each case. Of the 267 adenocarcinomas, detailed clinicopathological findings could be obtained in 128 cases. The definitions used for stage grouping, histological classification (grade of atypia) and depth of tumor invasion were based on the criteria of the Japanese Research Society for Cancer of the Colon and Rectum [10]. Depth of tumor invasion was classified as: m, mucosa including muscularis mucosae; sm, submucosa; mp, muscularis propria. The stage groups were: 0, lesion limited to m and no metastasis; 1, lesion confined to sm and/or mp and no metastasis; 2, invasion deeper than mp but limited to the bowel wall and no metastasis, 3, extension to extracolorectal tissue and/or regional lymph node metastasis; 4, metastasis to distant lymph node or organ and/or peritoneal dissemination regardless of depth of invasion. Informed consent was obtained from all subjects.

A monoclonal antibody to cyclin E (14591A) was purchased from Pharmingen (San Diego, USA). We have confirmed that this antibody reacts specifically with cyclin E protein in Western blotting and immunohistochemistry [1]. Anti-p53 polyclonal antibody (CM-1) and anti-Ki-67 monoclonal antibody (MIB-1) were obtained from Novocastra (Newcastle upon Tyne, UK) and Medical and Biological Laboratories (Nagoya, Japan), respectively.

A modification of the immunoglobulin enzyme bridge technique (ABC method) was used, as described elsewhere [26]. Deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block the endogenous peroxide activity. In the case of cyclin E and Ki-67, microwave pretreatment in citrate buffer was performed for 10×3 min to retrieve the antigenicity. The sections were then incubated with normal horse serum (diluted 1:20) for 30 min to block the nonspecific antibody binding sites. The sections were treated consecutively at room temperature with anti-cyclin E antibody (diluted 1:200), anti-p53 antibody (diluted 1:1000) or anti-Ki-67 antibody (diluted 1:100) for 90 min, biotinylated anti-mouse or rabbit IgG horse serum (diluted 1:100, Vector, Burlingame, USA) for 30 min, and avidin DH-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector) for 30 min. Peroxidase staining was performed for 10–15 min using a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were weakly counterstained with 0.1% hematoxylin.

All the immunostained slides were observed by three pathologists (W. Y., H. Y., and F. S.) independently to make the grading as objective as possible. After agreement on grade by the three, we examined the other findings, but only nuclear staining was regarded as positive for all three molecules.

The immunoreactivity was graded as – to +++ according to the number of cells stained and, in case of cyclin E, the intensity of the reaction in individual cells was taken into account. Grades were defined as follows. For cyclin E, –, almost no positive cells; +, 5–25% of tumor cells showed weak to moderate immunoreactivity; ++, 25–50% of tumor cells showed moderate immunoreactivity or 10–50% of tumor cells showed intense immunoreactivity; +++, over 50% of tumor cells showed intense immunoreactivity. For p53 and Ki-67, –, almost no positive cells; +, 5–25% of tumor cells showed immunoreactivity; ++, 25–50% of tumor cells showed immunoreactivity; +++, over 50% of tumor cells showed immunoreactivity. Grades ++ and +++ were regarded as strongly positive. The most prominent area of immunostaining was estimated in cases where immunoreactive cells were not equally distributed throughout the tumor.

Results

The incidence of cyclin E expression in colorectal adenomas and adenocarcinomas is summarized in Table 1. Of the 358 adenomas, 91 (25%) contained cyclin E-positive tumor cells. Adenomas were classified into three groups (adenoma with mild atypia, moderate atypia and severe atypia) according to the cellular and structural atypia [10] and analyzed the relation with the expression of cyclin E. Cyclin E-positive tumor cells were detected in 12% of adenomas with mild atypia, 21% of adenomas with moderate atypia and 31% of adenomas with severe atypia, the correlation being significant ($P<0.01$). The immunoreactivity was localized in the nuclei of the adenoma cells (Fig. 1a).

Cyclin E-positive tumor cells were observed in 45 (46%) of the 98 adenocarcinomas in adenomas and 104 (62%) of the 169 adenocarcinomas without an adenoma component, the incidence being significantly different ($P<0.05$). The incidence of strongly positive cases was significantly ($P<0.01$) higher both in the adenocarcinomas in adenomas (18%) and in the adenocarcinomas without adenoma component (21%) than in the adenomas (5%). As shown in Fig. 1b, c, cyclin E immunoreactivity was also localized in the nuclei of the carcinoma cells. Most of the adenocarcinomas were well differentiated or moderately differentiated, and no difference in the incidence of cyclin E expression was found by histological types.

We then analyzed the correlation of cyclin E expression with clinicopathological findings in the 128 carcino-

Table 1 Expression of cyclin E in colorectal adenomas and adenocarcinomas

Histological types	No. of cases	Cyclin-E-positive lesions	
		Positive[a]	Strongly positive[a]
Adenoma	358	91 (25%)	17 (5%)
Mild[b]	42	5 (12%)[c]	0 (0%)
Moderate	116	24 (21%)	3 (3%)
Severe	200	62 (31%)	14 (7%)
Adenocarcinoma in adenoma	98	45 (46%)[d]	18 (18%)[e]
Adenocarcinoma	169	104 (62%)[d]	35 (21%)[e]

[a] *Positive* grades +, ++ and +++, *strongly positive* grades ++ and +++

[b] Grade of atypia was classified into three according to the criteria of the Japanese Research Society for Cancer of the Colon and Rectum

[c] The incidence was significantly correlated with the grade of atypia ($P<0.05$, by Chi-square test)

[d] The incidence was significantly higher than that of adenomas ($P<0.01$, by Chi-square test). The incidence differed significantly between the two ($P<0.05$, by Chi-square test)

[e] The incidence was significantly higher than that of adenomas ($P<0.01$, by Chi-square test)

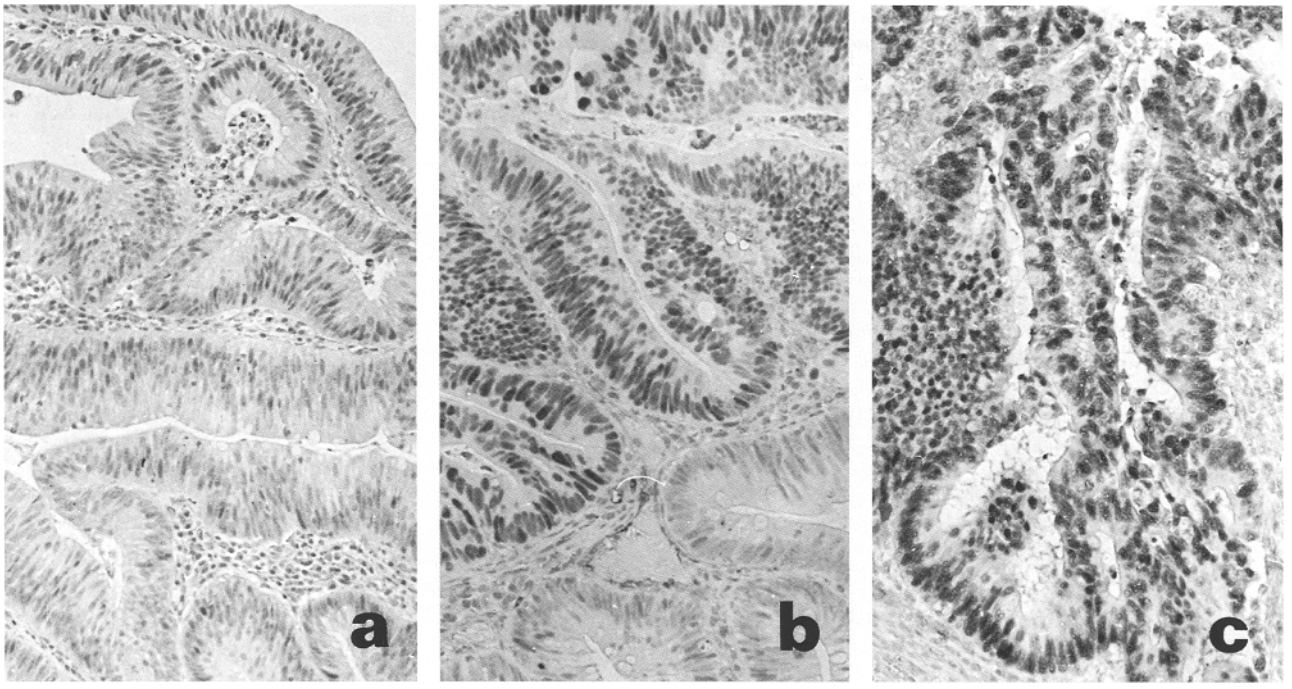

Fig. 1a–c Immunostaining of cyclin E in colorectal adenoma and adenocarcinoma. **a** Tubular adenoma with severe atypia. Many adenoma cells are weakly positive to cyclin E (staining grade +; ×160). **b** Adenocarcinoma in adenoma (well-differentiated tubular adenocarcinoma). Many carcinoma cells express cyclin E strongly (staining grade ++), while a few adenoma cells show faint immunoreactivity to cyclin E (staining grade –). Cyclin E expression is localized in the nuclei. (×200). **c** Advanced-stage adenocarcinoma. Intense immunoreactivity to cyclin E is observed in most of carcinoma cells. (Staining grade +++; ×200)

ma cases obtained by surgery or endoscopic removal in which full information could be obtained. As shown in Table 2, the incidence of cyclin E expression was significantly higher in stages 1 (lesion confined to submucosa and/or muscularis propria and no metastasis) and 2 (invasion deeper than muscularis propria but limited to the bowel wall and no metastasis) carcinoma than in stage 0 (lesion limited to the mucosal layer and no metastasis) carcinoma ($P<0.01$). However, the incidence of cyclin E expression in stages 3 and 4 carcinoma (metastases in lymph nodes or distant organs regardless of depth of invasion) was lower than that in stages 1 and 2 carcinoma. For the depth of tumor invasion, a similar correlation was detected. The expression of cyclin E was most prominent in the carcinomas that involved submucosa and muscularis propria but not deeper layers. The carcinomas with lymph node metastasis expressed less cyclin E than those without.

We next examined the relationship between cyclin E expression and proliferative activity of colorectal carcinomas. Since the cell population detected by Ki-67 antibody is referred to as the growth fraction (cells in G1, S, G2, M), Ki-67 antigen expression was analyzed as a proliferative marker [24]. Table 3 shows the relationship between cyclin E and Ki-67 expression. As the grades of Ki-67-immunoreactivity increased, those of cyclin E im-

Table 2 Correlation between cyclin E expression and clinocopathological findings of colorectal carcinoma

	No. of cases	Cyclin-E-positive cases	
		Positive[a]	Strongly positive[a]
Stage[b]			
0	86	30 (35%)	15 (17%)
1	18[c]	13 (72%)[e]	8 (44%)[f]
2	7	5 (71%)[e]	3 (43%)[f]
3	7	2 (29%)	1 (14%)
4	10	2 (20%)	0 (0%)
Depth of invasion[b]			
m	86	30 (35%)	15 (17%)
sm, mp	19[d]	13 (68%)[g]	8 (42%)[h]
Beyond mp	23	9 (39%)	4 (17%)
Lymph node metastasis			
Negative	115	50 (43%)[i]	27 (23%)
Positive	13	2 (15%)	0 (0%)

[a] *Positive* grades +, ++ and +++, *strongly positive* grades ++ and +++
[b] According to the criteria of the Japanese Research Society for Cancer of the Colon and Rectum: *m* mucosa including muscularis mucosae, *sm* submucosa, *mp* muscularis propria; *stage 0* lesion limited to m and no metastasis, *1* lesion confined to sm and/or mp and no metastasis, *2* invasion deeper than mp but no entension to extracolorectal tissue and no metastasis, *3* extension to extracolorectal tissue and/or regional lymph node metastasis, *4* metastasis to distant lymph node or organ and/or peritoneal dissemination regardless of depth of invasion
[c] Being 16 carcinomas limited to sm and 2 carcinomas invading mp
[d] Being 17 carcinomas limited to sm and 2 carcinomas invading mp
[e] The incidence was significantly higher in carcinomas of stages 1 and 2 than in those in stage 0 as well as in stages 3 and 4 ($P<0.01$, by Chi-square test)
[f] The incidence was significantly higher in stages 1 and 2 than in stage 0 ($P<0.01$, by Chi-square test)
[g] The incidence was significantly higher in carcinomas with sm or mp invasion than in those confined to m ($P<0.01$, by Chi-square test)
[h] The incidence was significantly higher in carcinomas with sm or mp invasion than in those within m ($P<0.05$, by Chi-square test)
[i] The incidence was significantly higher in carcinomas without metastasis than in those with metastasis ($P<0.05$, by Fisher's exact test)

Table 3 Correlation between cyclin E and Ki-67 in colorectal carcinomas

		Ki-67-immunoreactivity[a]			Total
		–	+	++, +++	
Cyclin E immunoreactivity[a]	–	24 (20%)	35 (28%)	64 (52%)	123 (49%)
	+	0 (0%)	23 (30%)	54 (70%)	77 (30%)
	++, +++	0 (0%)	11 (20%)	43 (80%)	54 (21%)
	Total	24 (9%)	69 (27%)	161 (63%)	254

[a] Immunoreactivity was graded –, +, ++ and +++ as described in Materials and methods. The expression of cyclin E and Ki-67 was significantly correlated ($P<0.01$, by Chi-square test)

Table 4 Correlation between Ki-67 antigen expression and clinicopathological findings of colorectal carcinoma

	No. of cases	Ki-67 positive cases	
		Positive[a]	Strongly positive[a]
Stage[b]			
0	86	68 (79%)	43 (50%)
1	18[c]	17 (94%)	12 (67%)
2	7	7 (100%)	6 (86%)
3	7	6 (86%)	4 (57%)
4	10	9 (90%)	7 (70%)
Depth of invasion[b]			
m	86	68 (79%)	43 (50%)
sm, mp	19[d]	18 (95%)[e]	13 (68%)[e]
Beyond mp	23	21 (91%)[e]	16 (70%)[e]
Lymph node metastasis			
Negative	115	95 (83%)	64 (56%)
Positive	13	12 (92%)	8 (62%)

[a] *Positive* grades +, ++ and +++, *strongly positive* grades ++ and +++
[b] See footnote to Table 2
[c] Being 16 carcinomas limited to sm and 2 carcinomas invading into mp
[d] Being 17 carcinomas limited to sm and 2 carcinomas invading into mp
[e] The incidence was significantly higher in carcinomas invading deeper than sm than in those confined to m ($P<0.05$, by Chi-square test)

munoreactivity increased, the positive correlation being significant ($P<0.01$). A representative carcinoma case with the expression of cyclin E and Ki-67 is shown in Fig. 2. Simultaneous expression of cyclin E and Ki-67 was observed in many of the tumor cells. Table 4 is a summary of Ki-67 expression in colorectal carcinomas. The Ki-67-positive tumor cells in the carcinomas invading into submucosa or deeper were significantly greater in number than in those in carcinomas limited to the mucosa. However, unlike the expression of cyclin E, the incidence of Ki-67 expression was not reduced in advanced carcinoma cases, although a significant correlation between cyclin E and Ki-67 was detected.

The correlation between the expression of cyclin E and p53 protein was also examined in colorectal carcinomas. As shown in Table 5, a significant correlation between cyclin E and p53 was detected ($P<0.01$). Figure 3 illustrates a representative carcinoma case with simultaneous expression of cyclin E and p53. The expression of p53 and the clinicopathological information are summarized in Table 6. The correlation between p53 and clinicopathology is similar to that with Ki-67. Cases in stages 1–4 and carcinomas invading the submucosa or even

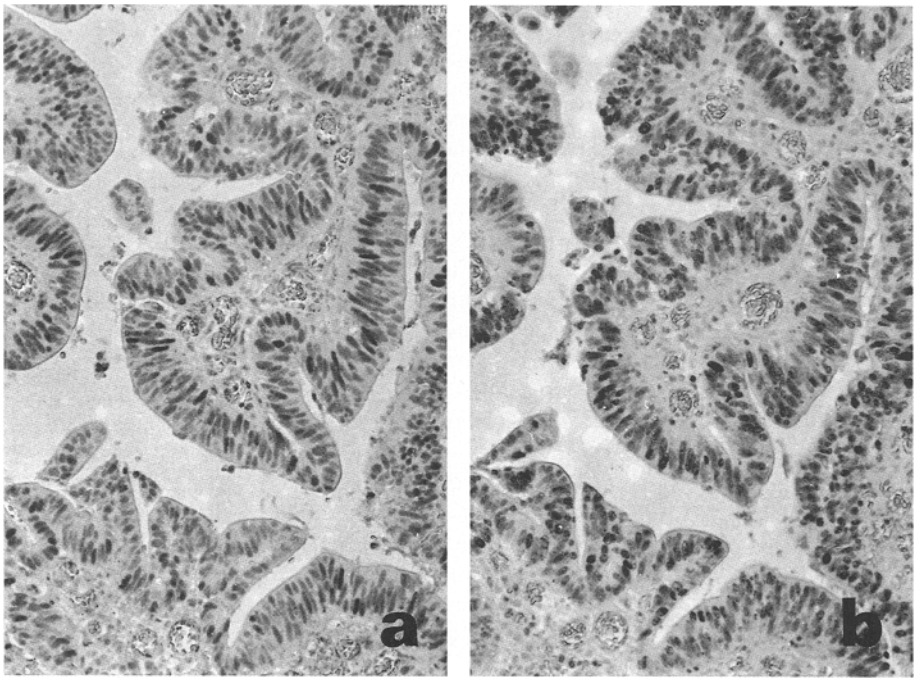

Fig. 2 Co-expression of **a** cyclin E and **b** Ki-67 in a colorectal carcinoma. Many carcinoma cells are positive to both cyclin E (staining grade ++) and Ki-67 (staining grade ++). (×180)

Table 5 Correlation between cyclin E and p53 in colorectal carcinomas

		p53 immunoreactivity[a]			Total
		–	+	++, +++	
Cyclin E immunoreactivity[a]	–	78 (64%)	13 (11%)	30 (25%)	121 (48%)
	+	32 (42%)	14 (18%)	31 (40%)	77 (31%)
	++, +++	19 (35%)	10 (19%)	25 (46%)	54 (21%)
	Total	129 (51%)	37 (15%)	86 (34%)	252

[a] Immunoreactivity was graded –, +, ++ and +++ as described in Materials and methods. The expression of p53 and cyclin E was significantly correlated ($P<0.01$, by Chi-square test)

deeper layer contained more p53-positive cells than stage 0 carcinomas and those limited to the mucosa. No reduction in the incidence of p53-positive tumors was detected in advanced cases.

Discussion

We have recently reported amplification of the cyclin E gene in 10% of colorectal carcinomas [15]. In the study concerned we could not detect the relationship between gene amplification and tumor progression, possibly because the number of cases with the amplification was so small. The main purpose of the present study was to clarify the role of cyclin E expression in the development and progression of colorectal carcinomas.

Table 6 Correlation between abnormal accumulation of p53 and clinocopathological findings of colorectal carcinoma

	No. of cases	p53-positive cases	
		Positive[a]	Strongly positive[a]
Stage[b]			
0	86	30 (35%)	18 (21%)
1	18[c]	10 (56%)[e]	9 (50%)[f]
2	7	4 (57%)[e]	4 (57%)[f]
3	7	4 (57%)[e]	4 (57%)[f]
4	10	5 (50%)[e]	5 (50%)[f]
Depth of invasion[b]			
m	86	30 (35%)	18 (21%)
sm, mp	19[d]	11 (58%)[g]	10 (53%)[h]
Beyond mp	23	12 (52%)[g]	12 (52%)[h]
Lymph node metastasis			
Negative	115	45 (39%)	32 (28%)
Positive	13	8 (62%)	8 (62%)[i]

[a] *Positive* grades +, ++ and +++, *strongly positive* grades ++ and +++
[b] See footnote to Table 2
[c] Being 16 carcinomas limited to sm and 2 carcinomas invading into mp
[d] Being 17 carcinomas limited to sm and 2 carcinomas invading mp
[e] The incidence was significantly higher in carcinomas of stages 1, 2, 3 and 4 than in those of stage 0 ($P<0.05$, by Chi-square test)
[f] The incidence was significantly higher in carcinomas of stages 1, 2, 3 and 4 than in those of stage 0 ($P<0.01$, by Chi-square test)
[g] The incidence was significantly higher in carcinomas invading deeper than sm than in those confined to m ($P<0.05$, by Chi-square test)
[h] The incidence was significantly higher in carcinomas invading deeper than sm than in those confined to m ($P<0.01$, by Chi-square test)
[i] The incidence was significantly higher in carcinoma with metastasis than in those without metastasis ($P<0.05$, by Chi-square test)

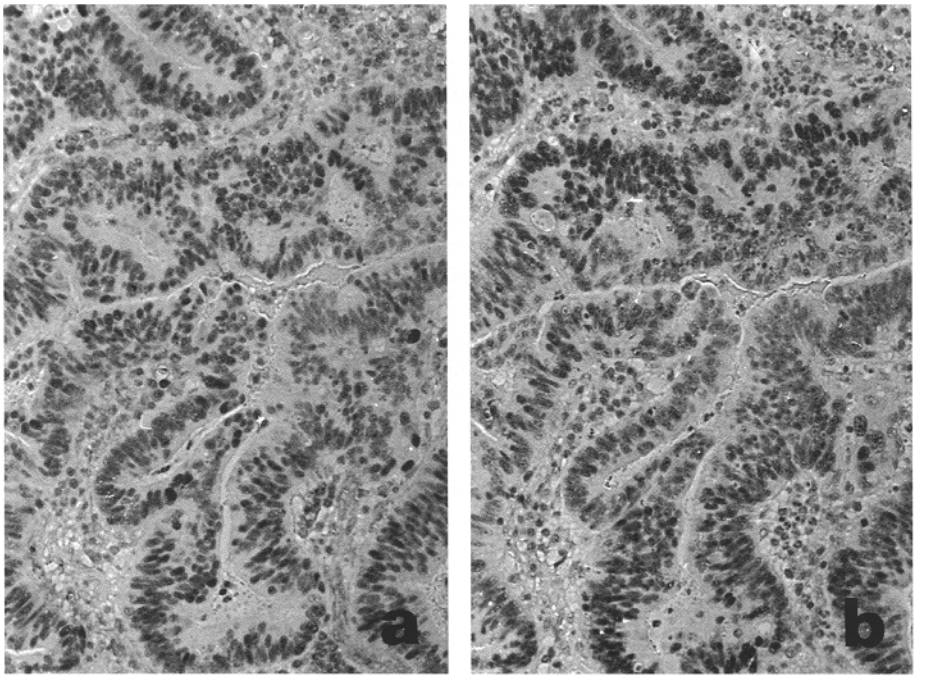

Fig. 3 Expression of **a** cyclin E and **b** p53 protein in a colorectal carcinoma. Many carcinoma cells are positive to p53 (staining grade +++) and show moderate immunoreactivity to cyclin E (staining grade ++). (×200)

We found the expression of cyclin E in 25% of colorectal adenomas and 46–62% of adenocarcinomas. The incidence of cyclin E-positivity was significantly higher in adenocarcinomas than in adenomas. An interesting correlation was found between the expression of cyclin E and cellular and structural atypia of adenomas, the expression of cyclin E becoming more frequent with increasing grade of atypia. These results suggest that overexpression of cyclin E may be involved in colorectal carcinogenesis at an early stage, although there is no direct evidence indicating that cyclin E acts as an oncogene. In adenomas, the expression of cyclin E did not differ with the histological pattern (tubular type or tubulo-villous type). Since our adenoma samples included only a small number of pure villous adenomas, the characteristics of cyclin E expression could not be detected in this type of adenoma.

Recently, we have found that in gastric carcinomas, cyclin E expression is frequently associated with lymph node metastasis and also with deeply invasive carcinomas [27]. However, a distinct relation between cyclin E expression and tumor progression was detected in the case of colorectal carcinoma. The incidence of cyclin E-positivity was significantly higher in stage 1 carcinomas (no metastasis and confined to submucosa and/or muscularis propria) and in stage 2 carcinomas (no metastasis and invasion deeper than muscularis propria but limited to the bowel wall) than in stage 0 carcinomas (no metastasis and limited to the mucosa). As to the depth of tumor invasion, carcinomas invading the submucosa and muscularis propria expressed cyclin E most prominently. In contrast, advanced cases, such as stages 3 and 4 carcinoma, deeply invasive carcinoma and carcinoma with metastasis, expressed less cyclin E. These observations suggest that overexpression of cyclin E in colorectal carcinomas may relate to early progression but not to later invasive events. Cyclin E may contribute to local proliferation and invasion and to penetration through muscularis mucosae.

The expression of cyclin E was significantly correlated with the proliferative activity of the tumor cells monitored by Ki-67, although Ki-67 expression was not reduced in advanced cases. More than 80% of the cases fell into stages 0, 1 and 2. Therefore, the significant correlation obtained here demonstrates an association of cyclin E and proliferative activity in the relatively early stage of colorectal carcinomas.

We also found a significant correlation between the expression of cyclin E and p53 protein. We examined p53-immunoreactivity in gastric and colorectal carcinomas whose genetic status of p53 was known [2, 28] and found a good agreement between p53 immunostaining and *p53* gene abnormalities in more than 70% of the carcinomas. In our staining conditions (without microwave pretreatment), positive staining for p53 might reflect abnormal accumulation of p53 encoded by a mutated p53. Unlike cyclin E expression, abnormal accumulation of p53 was found in 50–57% of colorectal carcinomas of stages 1–4 without any reduction in the incidence with advancing stage. Therefore, the association of p53 and cyclin E should also be indicative of the early stages (stages 0, 1 and 2). Wild-type, but not mutant, p53 has been shown to regulate the cell cycle negatively by induction of CDK inhibitor, p21 [5]. Alterations (loss of heterozygosity and mutation) to the p53 gene occur in 60–80% of colorectal carcinomas [2], and the cyclin E–CDK2 complex is one of the most important targets of p21. Since p21 inhibits the function of cyclin E-CDK2 [25], it is interesting to speculate whether abnormal p53 induces the overexpression of cyclin E through p21 repression in colorectal carcinomas.

Deregulation of multiple cyclins, CDKs and CDK inhibitors contributes to carcinogenesis by encouraging uncontrolled cell proliferation. An increased body of evidence suggests that the cyclins involved in carcinogenesis may have organ specificity. For example, amplification and overexpression of cyclin D1 is frequently associated with carcinomas of the esophagus and breast, but not of the stomach and colon [1, 3, 11], while amplification of cyclin E is exceptional in esophageal carcinomas [16]. Expression of cyclin E may be implicated in early progression of colorectal carcinomas, while abnormalities of cyclin E in gastric carcinomas confer malignant behavior, such as deep invasion and metastasis [1]. It seems likely that the role of cyclin E in the development and progression of cancer may have organ specificity.

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